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Authors

Chakedis, J
French, R
Babicky, M
et al.

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A novel protein isoform of the RON tyrosine kinase receptor transforms human pancreatic duct epithelial cells

Jeffery Chakedis, **Randall French**, **Michele Babicky**, **Dawn Jaquish**, **Haleigh Howard**, **Evangeline Mose**, **Raymond Lam**, **Patrick Holman**, **Jaclyn Miyamoto**, **Zakk Walterscheid**, and **Andrew M. Lowy**

Department of Surgery, Division of Surgical Oncology, Moores Cancer Center, University of California, San Diego, La Jolla, CA

Abstract

The *MST1R* gene is overexpressed in pancreatic cancer producing elevated levels of the RON tyrosine kinase receptor protein. While mutations in *MST1R* are rare, alternative splice variants have been previously reported in epithelial cancers. We report the discovery of a novel RON isoform discovered in human pancreatic cancer. Partial splicing of exons 5 and 6 (P5P6) produces a RON isoform that lacks the first extracellular immunoglobulin-plexin-transcription (IPT) domain. The splice variant is detected in 73% of pancreatic adenocarcinoma patient derived xenografts and 71% of pancreatic cancer cell lines. Peptides specific to RON P5P6 detected in human pancreatic cancer specimens by mass spectrometry confirms translation of the protein isoform. The P5P6 isoform is found to be constitutively phosphorylated, present in the cytoplasm, and it traffics to the plasma membrane. Expression of P5P6 in immortalized human pancreatic duct epithelial (HPDE) cells activates downstream AKT, and in human pancreatic epithelial nestin-expressing (HPNE) cells activates both the AKT and MAPK pathways. Inhibiting RON P5P6 in HPDE cells using a small molecule inhibitor BMS-777607 blocked constitutive activation and decreased AKT signaling. P5P6 transforms NIH3T3 cells and induces tumorigenicity in HPDE cells. Resultant HPDE-P5P6 tumors develop a dense stromal compartment similar to that seen in pancreatic cancer. In summary, we have identified a novel and constitutively active isoform of the RON tyrosine kinase receptor that has transforming activity and is expressed in human pancreatic cancer. These findings provide additional insight into the biology of the RON receptor in pancreatic cancer and are clinically relevant to the study of RON as a potential therapeutic target.

Keywords

RON; tyrosine kinase; isoform; pancreatic cancer; alternative splicing; tumorigenesis

Corresponding Author: Andrew M. Lowy, MD, UC San Diego Moores Cancer Center, 3855 Health Sciences Drive, Mail Code: 0987, La Jolla, CA 92093-0987, 858-822-2124 office, 858-228-9787 fax, alowy@ucsd.edu.

Conflicts of Interest

The authors have no conflicts of interest to disclose.

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INTRODUCTION

While there have been recent advances in the use of cytotoxic therapy to treat pancreatic cancer, molecularly targeted treatments have yet to make an impact in the clinic. To develop effective therapeutics for a disease with few treatment options, the molecular structure and function of proteins expressed in pancreatic cancer must be identified and understood. RON is a growth factor receptor belonging to the MET family which has been suggested as a putative target for cancer therapeutics [1, 2]. RON signaling impacts not only the cancer cell but is a regulator of the tumor microenvironment thru its effects on tumor-associated macrophages [3, 4]. Currently, a monoclonal antibody (RON8, ImClone) and small molecule inhibitors are in Phase 1 trials and various stages of pre-clinical development, respectively [5, 6].

RON is expressed at minimal levels in normal pancreatic tissue, but is overexpressed in pancreatic and epithelial cancers of the breast, colon, lung, and prostate[2]. During multistep carcinogenesis from low to high grade pancreatic intraepithelial neoplasia (PanIN), RON is increasingly expressed [7]. Overall the protein is expressed in 69-96% of pancreatic cancer specimens [7, 8]. Upon receptor activation, the MAPK and AKT signaling pathways are but two of the downstream targets activated to promote survival, motility, tumor growth and invasion of cancer cells *in vivo* [9-11].

The full length RON protein is 1400 amino acids and is translated as a single pro-protein which is cleaved into an α and β subunit to form the extracellular sema domain[12]. The sema domain recognizes the receptor ligand, Macrophage Stimulating Protein (MSP), and binding subsequently activates the intracellular tyrosine kinase domain [13]. RON is activated when two receptor molecules form a homodimer and bind one MSP ligand molecule [14]. When the receptor is overexpressed, interactions between the extracellular portions of the receptor cause dimerization and is hypothesized to be the mechanism of ligand independent activation.

RON is rarely mutated, but alternative splicing events are common as the gene transcript is increasingly overexpressed. Approximately eight different RON isoforms have been described in various epithelial cancer types [15]. Exon skipping is the most common splicing event, creating RONΔ165 and RONΔ160 where exon 11 and exons 5+6 are skipped, respectively [16, 17]. A short form of RON is produced by an alternative promoter and transcription start site in intron 10, producing a protein that lacks the entire extracellular domain [18]. In breast cancer, short form RON overexpression specifically activates the PI3K signaling pathway which results in increased tumor growth, epithelial to mesenchymal transition (EMT), and increased metastasis [19]. Each of these three isoforms is constitutively phosphorylated and contributes to an increasingly invasive phenotype.

Several preclinical studies have demonstrated robust antineoplastic activity following RON inhibition and form the basis for development of therapeutics targeted to the receptor [9, 20, 21]. Understanding the biology of RON isoform splice variants will allow us to more effectively evaluate current therapeutics and develop new, targeted therapies. In this study we identified a novel RON isoform produced by alternative splicing of exons 5 and 6.

Alternative 5’ and 3’ splice sites produce a transcript and protein product that omits a portion of exons 5 and 6 (P5P6). We demonstrate that P5P6 protein is expressed in human pancreatic cancer specimens and it transforms immortalized human pancreatic ductal epithelial cells such that they become tumorigenic *in vivo*.

RESULTS

Identification of a novel RON splice variant in pancreatic cancer

Previous studies in colon cancer and glioma revealed that RON isoforms are often generated based on alternatively spliced transcripts between exons 4 and 7 [17, 22, 23]. As no study to date has explored alternative splicing of RON in human pancreatic cancer, we sought to determine if those alternative splicing events previously identified were present. Initially, we analyzed RNA from a human pancreatic cancer by end point PCR to amplify a region of RON between exons 4 and 7 (Figure 1A). Sequencing the resulting bands confirmed that they were specific for the full length protein, exon 6 skipping (RONΔ90), partial exon 5 and partial exon 6 skipping (P5P6), and exon 5 and 6 skipping (RONΔ160). To our knowledge, the RON P5P6 splice variant has not previously been identified in any tumor type. This novel isoform is produced by an alternative 5’ splice site within exon 5 and an alternative 3’ splice site within exon 6. Splicing is in frame and compared to the full length protein produces a transcript which has skipped 195 nucleotides (Figure 1B).

The P5P6 protein product is 1335 amino acids, yielding a molecular weight of 145 kDa for the pro-P5P6 and 112 kDa for the cleaved β-subunit. Omission of this 65 amino acid sequence corresponds to loss of the first Immunoglobulin-Plexin-Transcription (IPT) domain (Figure 1C). A similar region is also deleted in the RON variant Δ160 that is expressed in colon and breast cancer [17, 24]. Three cysteine residues are removed by deletion of these amino acids that function to produce intermolecular disulfide bonds. Deletion of these cysteine residues is functionally significant and in RONΔ165 produces unbalanced bonds and RON oligomer formation [16].

We next determined whether the P5P6 isoform could be identified in normal pancreatic tissue and what the prevalence of P5P6 expression is in pancreatic cancer. First we performed PCR using primers specific for the P5P6 junction as well as total RON transcripts using primers directed at the C-terminus (Figure 2A). These studies revealed that P5P6 transcript could not be identified in normal pancreas, but was detected in 73% of PDX and 71% of cell lines (Figure 2B).

Next we quantified the amount of RON mRNA being produced using absolute quantitative Real Time PCR in both patient-derived xenografts (PDX) and pancreatic cancer cell lines. There are no commercial antibodies specific to isoforms of RON therefore we could not quantitate isoform protein expression using traditional methods. Plasmids containing full length RON and P5P6 transcripts were used to generate standard curves (Supplementary Figure S1). Efficiency was calculated at 106% for RON c-terminal primers and 91% for the P5P6 primer pair. We found that whole normal pancreas expresses 67 copies of total RON transcripts per 73 ng of RNA and reaffirmed the absence of P5P6 expression (Figure 2C). We demonstrated quantitatively for the first time that the magnitude of RON overexpression

at the RNA level is 150-400 times that present in normal pancreatic tissue. Similarly, pancreatic cancers also generated high copy numbers of P5P6 transcript ranging from 1304 – 4215 copies per 73 ng of RNA or 20-70 fold greater abundance than total RON transcript seen in normal tissue. Cell lines that overexpress RON (Bx-PC3, AsPC1, 79E, 34E) also generated high copy numbers of P5P6 transcript.

P5P6 protein can be detected by mass spectrometry

While we have demonstrated that the P5P6 transcript was abundant in pancreatic cancer specimens, any putative biologic consequences of P5P6 are predicated on translation to a functional protein. We first utilized a non-specific RON antibody (C-20) to the c-terminal portion of the β chain to determine RON expression pattern in PDXs. Although some PDXs give a western blot signal around 115 kDa (Red arrow in Figure 3A), there is no certainty that this corresponds to the P5P6 protein (Figure 3A).

We therefore utilized mass spectrometry to investigate the presence of P5P6 protein in pancreatic cancer based on the finding that RON isoforms produce unique peptide fragments after digestion with trypsin (Figure 3B). Each isoform protein sequence was analyzed by the ExPASy PeptideCutter tool that predicts peptides generated after trypsin digestion [25]. Immunoprecipitation of two PDXs (1713 and 1444) was performed with an antibody specific for the C-terminal portion of RON (E9) and the N-terminal portion (RON8). There was adequate protein pulled down as seen on a stained gel and RON peptides were the most common in the sample (Supplementary Figure S2). Ultimately, PDX 1713 contained a peptide unique for the P5P6 protein (Figure 3C). None of the remaining peptides that were identified were isoform specific (Figure 3D). This proves that generation of the P5P6 transcript results in translation to P5P6 protein in pancreatic cancer. P5P6 protein identification suggests it is present in relatively high abundance compared to other isoforms whose unique peptides could not be detected using this methodology.

The P5P6 isoform induces oncogenic phenotypes

To understand its effects on cancer cell biology we cloned the RON P5P6 cDNA into full length RON mRNA and attached it to enhanced Green Fluorescent Protein (eGFP). This recreated the partial exon 5 and 6 splicing and created the P5P6-eGFP (P5P6e) fusion protein. This was transfected into HPDE, Human Pancreatic Nestin-expressing Epithelial (HPNE), COS-1 (Cos), and MiaPaca2 (MP2) cell lines using a lentiviral vector. HPDE expresses low levels of full length RON while HPNE, Cos, and MP2 were chosen as they do not express detectable RON protein. RON P5P6e expression and phosphorylation levels were stable throughout all experiments. HPDE P5P6e expressing cell lines displayed no obvious change in morphology in culture (Figure 4A). The P5P6e protein primarily localizes to the cytoplasm in a reticular pattern surrounding the nucleus and to the plasma membrane (arrows, Figure 4B). We also created an untagged version RON P5P6 cDNA and expressed it in HPDE cells to rule out any effects of the fusion protein on oncogenic phenotypes (Supplementary Figure 3).

Western blot of the stably transduced cell lines show that the P5P6 protein is detectable by conventional RON β antibodies (Figure 4B). In all cell lines, it produces a similar pattern of

two bands which are an uncleaved pro-form (172 kDa) and a cleaved β chain (139 kDa). The HPDE P5P6e cell line expresses somewhat higher levels of the fusion protein and subsequent degradation products can be seen (shown is a less exposed blot, red arrows Figure 4C). Immunoblot for the α subunit of RON confirms that P5P6 is cleaved into separate subunits similar to the full length protein. Immunoprecipitation with RON β antibody and immunoblot with phosphotyrosine (pY) shows that the RON P5P6 protein is constitutively phosphorylated. In the HPDE cell line the degradation products are also still phosphorylated as the N terminal part of the β chain is cleaved (red arrows). Importantly, the level of P5P6e protein expressed in the HPDE and HPNE cell lines is similar to the overexpression of total RON in 3 pancreatic cancer cell lines and 3 PDXs (Figure 4D). This demonstrates these cell lines are producing physiologic levels of RON protein comparable to that seen in human pancreatic cancers and cell lines.

In HPDE cells AKT is active at baseline and RON P5P6 increased phosphorylation by 2 fold. However there were no significant effects on ERK phosphorylation (Figure 5A). Parental HPNE cells do not express RON, however when RON P5P6e is expressed both the AKT and MAPK pathways are activated. In contrast, in MP2 cells there was a modest increase in MAPK signaling while minimal changes were noted in Cos cells, strongly suggesting the effects of P5P6 are cell and context specific. We also noted that in HPDE cells, MSP stimulation did not change phosphorylation of P5P6 and appeared to alter downstream signaling only through the native full-length RON receptor (Figure 5B).

RON is under investigation as a therapeutic target and BMS-777607 is a MET family inhibitor which effectively inhibits RON signaling in pancreatic cancer [30]. Treatment with BMS-777607 at 3 μmol/L completely inhibited phosphorylation in the HPDE P5P6e line that could not be rescued with MSP stimulation (Figure 5C). Signaling through AKT was significantly reduced and there was no effect on MAPK. Since BMS-777607 is not a highly specific RON inhibitor, the effects on signaling are obviously not necessarily secondary to P5P6 inhibition alone.

RON P5P6 isoform signaling is distinct from RONΔ160 and full length RON

The P5P6 isoform is most similar in structure to RONΔ160 that results from complete skipping of exons 5 and 6. The mechanism of RONΔ160 induced oncogenesis involves accumulation of β-catenin, which is required for isoform mediated cell transforming activities [26]. We observed no accumulation of total β-catenin in the P5P6e expressing cell lines (Figure 5D). STAT3 is a transcription factor implicated in multiple stages of KRAS driven cancer progression [27]. STAT3 is activated when full length RON is stimulated with its ligand MSP, and high levels of STAT3 phosphorylation were correlated with high levels of RON expression in gastroesophageal adenocarcinoma [28]. In contrast, we found that RON P5P6 expression does not activate STAT3 signaling (Figure 5E). Conversely, in the Cos and MP2 lines there is a loss of total STAT3 and a concordant decrease in phosphorylation. These experiments suggest that P5P6 signaling is mechanistically distinct from both RON Δ160 and the full length protein as the β-catenin and STAT3 pathways are not downstream targets.

P5P6 is transforming and induces oncogenic phenotypes

We next performed functional assays to determine if P5P6e signaling induced an oncogenic phenotype. We found no differences in proliferation between the HPDE P5P6e, MP2 P5P6e and their parental lines, a finding consistent with prior studies of RON signaling in pancreatic cancer cells (Supplementary Figure S4A). Wound closure assay showed HPDE P5P6e expressing cells induced significantly greater wound closure at all time points. At 72 hours the extent of wound closure was 93% and 66% ($P < .001$) for the HPDE P5P6e expressing and parental HPDE line respectively (Figure 6A). Interestingly, in the poorly differentiated MP2 cell line, P5P6e cell line slightly decreased wound closure at all times points again suggesting the contextual relevance of P5P6 activity (Supplementary Figure S4B). We have previously demonstrated that RON signaling activates VEGF production by pancreatic cancer cells via the MAPK pathway [29]. While we noted a 36% increase (263 pg/mL vs 194 pg/mL, $P = 0.011$) in VEGF secretion in HPDE P5P6e compared to the parental line (Supplementary Figure 5), this increase is small and likely is not biologically significant.

Finally, we utilized a colony forming assay to determine whether P5P6 expression could transform NIH3T3 cells (Figure 6B). P5P6 expression significantly increased colonies formation compared to empty vector, yet the effect was more modest than expression of mutant KRAS. Collectively, these experiments reveal that P5P6 has transforming activity and enhances migration in human pancreatic duct cells. The effects on migration appear to be specific to the context as they are not observed either in a non-pancreatic epithelial cell line (Cos) or in the poorly differentiated pancreatic cancer cell line (MP2).

RON P5P6 expression is tumorigenic *in vivo*

The previous series of assays suggested that expression of P5P6e in HPDE cells induced both molecular and phenotypic alterations. To determine if these alterations reached a threshold sufficient to alter *in vivo* biology, we used an orthotopic cell-line derived xenograft model. The HPDE cell line has previously been shown to be non-tumorigenic in SCID mice [31]. We injected one million HPDE or HPDE P5P6e cells into murine pancreata as an orthotopic xenograft. While the parental HPDE-derived xenografts failed to induce tumors in ten mice after 16 weeks, the HPDE P5P6e orthotopic injections resulted in tumor formation in six of eight mice that were palpable after 4 weeks (p-value 0.02, Figure 7A). Expression of the P5P6 eGFP fusion protein was evidenced by bright tumor fluorescence. Subcutaneous injection assays were also performed and produced identical results (Figure 7B). Orthotopic tumors had a mean weight and volume of 178 mg and 156 mm^3 (p-value 0.03, Figure 5C). One orthotopic tumor locally invaded the abdominal wall but we found no gross evidence of metastatic disease.

A hallmark of pancreatic cancer is the dense stromal component present within the tumors. We performed H&E staining and immunohistochemistry (IHC) on the P5P6e tumor sections to characterize tumor histology and protein expression (Figure 7D). Merged confocal images show the majority of the tumor was HPDE P5P6e expressing cells (Green, GFP), but that there was a significant stromal component to the tumors (Blue, DAPI). Ductal structures which express E cadherin (arrows, Figure 7D) but not the GFP fusion protein represent

residual clusters of host mouse pancreatic acinar cells. RON isoforms have been reported to promote epithelial to mesenchymal transition (EMT) but we did not observe significant morphologic evidence of P5P6 induced EMT either in vivo or in vitro. Cells maintained a round shape and expressed e-cadherin homogenously (Supplementary Figure S6).

DISCUSSION

The macrophage stimulating protein receptor 1 (*MST1R)* gene locus contains the gene for RON and its splice variants on chromosome 3p21 [32]. This region of the genome contains many oncogenes and is frequently altered in many epithelial malignancies [33]. During carcinogenesis as RON is increasingly expressed in pancreatic cancer there is subsequent generation of splice variants. In this study, we report the discovery of a novel RON isoform that is produced by partial skipping of exons 5 and 6 and is expressed in the majority of pancreatic cancers and pancreatic cancer cell lines. No previous reports have quantified levels of RON isoform production for comparison, however the amount of P5P6 transcript is 20-70 fold times the amount of total RON expressed in normal pancreas. Quantification of splice variants will be important in future studies to determine if there is isoform switching between cancer types or during oncogenic progression.

Detection of P5P6 protein is difficult as there are no specific antibodies and the size is similar to the wild type protein. Despite this, we were able to confirm expression of P5P6 protein in pancreatic cancer by detection of a specific peptide using mass spectrometry. To our knowledge this is the first demonstration of RON isoform protein expression in human cancer to be documented by a method other than immunoblotting. The P5P6 protein isoform has a truncation of the first IPT domain which may be a cause of its deregulation. RON shares domain structures with plexins, a family of semaphorin protein receptors [34]. Class B plexins can couple with RON and MET in the absence of ligand to activate the receptor and promote invasive activity [35]. Deletion of this domain will alter the proteins molecular structure leading to increased or decreased affinity for conventional RON binding partners. IPT domain deregulation may also contribute to constitutive activation of the protein through unbalanced cysteine residues. The P5P6 isoform traffics to the plasma membrane similar to the full length protein which allows for binding of ligand, interaction with cell surface receptors, and interaction with cytoplasmic binding partners. It is clear that P5P6 protein is phosphorylated in the absence of ligand and may be due to accumulation of P5P6 dimer formation and subsequent auto-phosphorylation.

Overexpression of P5P6 in the immortalized HPDE cell line resulted in increased AKT signaling, cell migration, and transformed the cell line to become oncogenic. The HPDE cell line has no mutations in the most commonly mutated genes in pancreatic cancer - *p53, KRAS, CDKN2A, or SMAD4*, though p53 is nonfunctional and there is loss of the *Rb* gene [31]. The HPDE and HPNE lines are the closest representation of a normal pancreatic epithelium available and is a limitation to this study. The context of P5P6 overexpression clearly matters as the biologic changes we observed in HPDE and HPNE differ in an unrelated cell line (Cos) and a mesenchymal pancreatic cell line (MP2). This raises the possibility that P5P6 expression may exert biologic effects early in oncogenesis rather than after the accumulation of multiple mutational events. This study has limitations in that

functional studies rely on P5P6 overexpression. However, total RON levels in P5P6 lines are similar to pancreatic cancer cell lines and human samples. Furthermore, our qPCR transcript quantification is an average of all cell types contained within the tumor. In pancreatic cancer stromal cells represent 50-90% of the tumor volume and physiologic levels of RON P5P6 transcripts in the malignant epithelial cells are likely much higher than we have detected [36].

The discovery of novel RON protein isoforms has potential translational impact. Full length RON expression has been shown to be a prognostic marker for several cancers, but not for resectable pancreatic cancer [28, 37]. Use of RON as a predictive or prognostic marker may require consideration of its isoforms to make valid correlations. RON P5P6 is constitutively active and does not need ligand to interact with other proteins or signal. Monoclonal antibody therapies that prevent binding of ligand to the receptor and subsequent activation may therefore be ineffective against some RON isoforms. Small molecules may therefore be more broadly effective in inhibiting RON and its isoforms. In fact, we found that BMS-777607 inhibited P5P6 phosphorylation and decreased AKT signaling. Our findings for the P5P6 isoform are consistent with previous studies that suggest alteration of the IPT domain structure effects RON function in profound ways. Therapies that target these interactions may be of interest as modulators of full length RON and RON isoform signaling.

In summary, this study identified a new RON isoform, P5P6, which is commonly expressed in pancreatic cancer. Overexpression of the isoform transforms NIH3T3 cells and immortalizes, non-tumorigenic human pancreatic ductal epithelial cells. Initial studies into the mechanism of its action imply signaling mechanisms distinct from the full length protein and a structurally similar isoform. Ultimately discovery of this isoform supports previous studies suggesting that RON isoform expression is important in cancer initiation and tumorigenesis. Therapeutics directed at the RON receptor will be most effectively evaluated if isoform activity is considered. Future work will be directed at further characterizing expression of RON isoforms in pancreatic cancer to understanding their functional significance and susceptibility to therapeutic agents.

MATERIALS AND METHODS

Human Tissues

All human tissues were collected and utilized in accordance with IRB approved and IACUC approved protocols at the University of California, San Diego. Tumor tissue was implanted into the pancreas of NOD SCID Gamma (NSG) mice as described previously [38, 39]. Patient Derived Xenografts (PDX) were established and passaged after they were at least 10mm in size. Low passage (<5) tumors were used for analysis.

PCR

Trizol (Invitrogen) was used to extract total RNA from PDXs. A cDNA library was created using SuperScript III Reverse Transcriptase (Invitrogen) and PCR used RedTaq DNA Polymerase (Sigma). The following primers were used:

RON Exon 4 to 7: Forward – GGCATGGCATTTCATGGGCT, Reverse - AGAGTCTGTCTGTAGGCACC

RON C-Terminal : Forward – TAGTGTCTGCACTGCTTGGG, Reverse - GCTGTTCTGGACGCACATTC

RON P5P6 specific: Forward – GGCAGTACAAGGCCTACCAA, Reverse - TCAGTCCCATTGACCAGCAC

Quantitative real-time PCR

All reactions used SsoFast EvaGreen Supermix (Bio-Rad), primers (375nM final concentration), 1 μL of cDNA (corresponding to 73 ng of RNA). Absolute quantification used methods previously described [40]. A plasmid containing the full length RON and RON P5P6e mRNA was used to generate a standard curve using RON c-terminal and P5P6 specific primers respectively. The equation DNA MW (g) = ((# of bp) × (660 g/mol/bp))/ 6.022×10^{23} particles/mol was used to convert plasmid size to weight. Weight was used to construct a serial dilution of 10^7 – 10^2 plasmid copies. Standards were run in duplicate on each plate used to analyze unknown samples and linear standard curve was generated (Supplementary Figure S1). The slope of the lines corresponds to the efficiency of the reaction using equation Efficiency = $(10^{-1/\text{slope}}-1) \times 100\%$.

Immunoprecipitation, Western Blot, and Immunohistochemistry

Pancreatic tumors of approximately 100mg or cultured cells at approximately 80 % confluency were used to make protein lysates in RIPA buffer. Immunoprecipitation (IP) was performed using RON C-terminal (E9, Santa Cruz) or RON N-terminal (RON8, ImClone). For western blot primary antibodies used are RON β (C20, Santa Cruz), RON α (EP1132Y, Abcam), Phosphotyrosine (4G10, Millipore), Actin (A2066, Sigma), Phospho-AKT (9271,Cell Signaling), AKT (9272, Cell Signaling), Phospho- p44/42 MAPK (9101, Cell Signaling), p44/42 MAPK (9102, Cell Signaling), Phospho-β-catenin (Tyr-654, ECM Biosciences), β-catenin (8480, Cell Signaling), Phospho-Stat3 (9138, Cell Signaling), Stat3 (4904, Cell Signaling). For immunohistochemistry the antibodies used were GFP (NB100-1770, Novus Biologicals) and E-cadherin (610182, BD). ImageJ program was used for densitometry.

Mass Spectrometry Sample preparation and LC-MS-MS

Immunoprecipitation was performed on 3 mg of protein with RON c-terminal (E9, Santa Cruz) and RON N-terminal (RON8, Imclone) then beads were submitted and sample preparation performed as previously described [41]. Trypsin-digested peptides were analyzed by high pressure liquid chromatography (HPLC) coupled with tandem mass spectroscopy (LC-MS/MS) using nano-spray ionization [42]. The nanospray ionization experiments were performed using a TripleTof 5600 hybrid mass spectrometer (ABSCIEX) interfaced with nano-scale reversed-phase HPLC (Tempo). The collected data were analyzed using MASCOT® (Matrix Sciences) and Protein Pilot 4.0 (ABSCIEX) for peptide identifications. RON isoform protein sequences are not normally included in the Protein Pilot analysis software and were supplemented in order to determine their detection.

Molecular Cloning and Lentiviral Transfection

The P5P6 protein transcript was created by recreating the P5P6 splicing event in the full length RON cDNA which had previously been attached to GFP. The region between exons 3 and 8 was amplified using the primers (F – GGGACCAGGTTTTCCAGGTACC and R – GGTACCTGGTTCCTGGACCTTCCAG) from a primary pancreatic cancer sample. The PCR amplicon was TA cloned using a PCR Cloning Kit (Qiagen) and competent DH5α E. Coli (Invitrogen). The full length RON+eGFP vector and the P5P6 containing vector were cut with specific endonucleases *HindII* and *KpnI* (NEB) and ligated using T4 DNA Ligase (NEB). DNA sequencing confirmed correct P5P6 sequence and the cDNA was then transferred into a lentiviral plasmid for transfection. After transfection selective puromycin media was used and the TE300 inverted fluorescence microscope (Nikon) was used to screen for green fluorescence.

Functional Assays

For scratch wound assay, 1×10^6 cells were plated in a 6-well dish and allowed to attach and grow to confluency. A 1 mL aspirating pipette tip was used to create 3 vertical scratches in each well. Images were taken at 4× magnification with Nikon TE300 inverted fluorescence microscope. Metamorph (Molecular Devices) analysis software was used to outline and determine wound area. Scratch assay was performed three separate times and all data combined. NIH 3T3 cells were stably infected with pCDH CMV empty vector, P5P6-eGFP, or KRAS G12V plasmid and plated. After 2 weeks cells were fixed, stained with crystal violet and a Keyence BZ-X710 microscope scanned the well and counted purple colonies.

Cell Lines and Orthotopic Injections

The Human Pancreatic Ductal Epithelial (HPDE) cell line was a generous gift from Dr. Ming Tsao and the University Health Network and cultured in Keratinocyte-SFM (Gibco) with epidermal growth factor and bovine pituitary extract, and antibiotic-antimycotic (Gibco)[43]. MiaPaca2 and Cos1 cell lines were obtained from ATCC and cultured in DMEM (Corning) supplemented with 10% fetal bovine serum (Sigma). HPNE cells were obtained from Dr. Klemke at UCSD and cultured in DMEM/Base F media (InCell) supplemented with 5% FBS. For cell injections 1×10^6 HPDE and HPDE P5P6e cells were resuspended in 10 μL of PBS and mixed with 10 μL of Matrigel (BD). NSG mice were injected orthotopically using a 30 gauge needle. Mice were monitored for signs of distress and disability throughout the experiment. After 12-16 weeks mice were sacrificed and tumors were harvested. Tumor volume was measure as: Volume = 1/2(length $\times$ width ^{2}). Mice and tumors were imaged using MVX10 (Olympus) camera and cellSens (Olympus) software.

Statistical Analysis

All data was analyzed using SPSS Statistics v.20 (IBM) and Prism v.6 (GraphPad Software). Functional assay values were averaged from each cell line per time point and compared using one way ANOVA.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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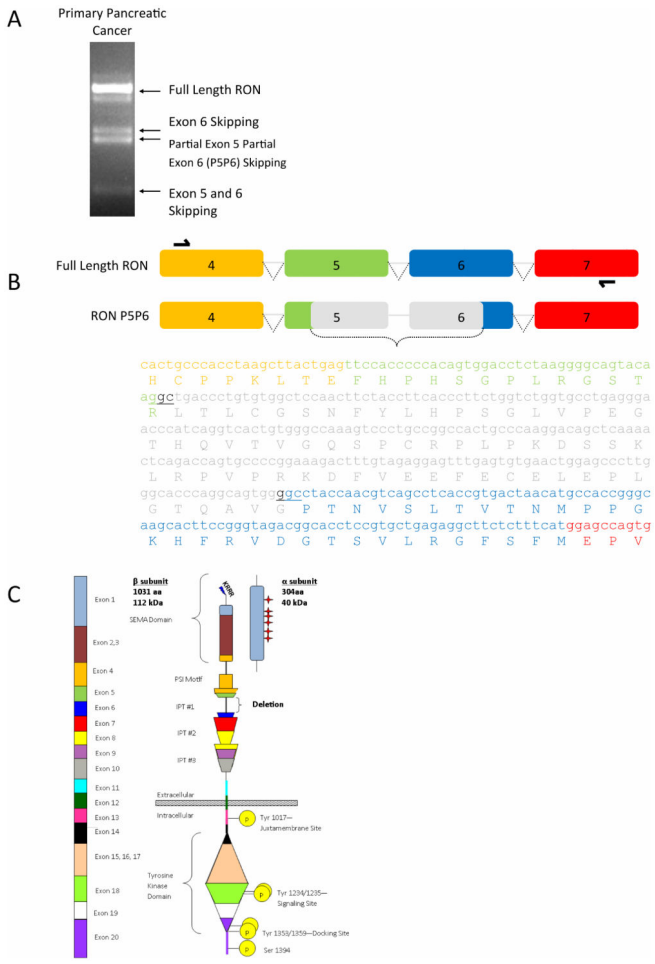
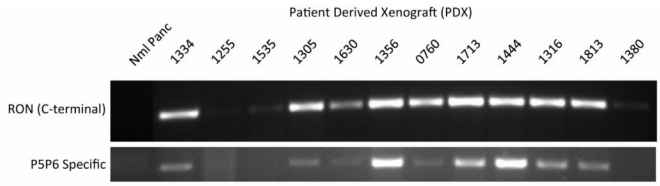


Figure 1.

Discovery of a novel RON isoform in human pancreatic cancer. A, A novel RON isoform created by alternative splicing of exons 5 and 6 is discovered in a pancreatic cancer specimen. B, The mRNA sequence of this splice variant contains a partial 5 and partial 6 (P5P6) exon splicing. C, There are 195 nucleotides omitted in the final protein resulting in a 65 amino acid deletion of the first Immunoglobin-Plexin-Transcription (IPT) domain. The P5P6 protein contains the extracellular Sema domain and intracellular tyrosine kinase domain similar to the full length RON protein.

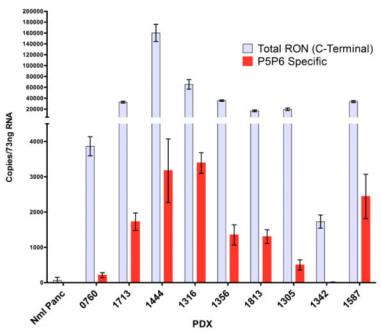
A



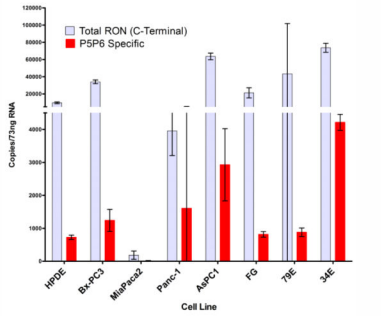
B

	PDX	Pancreatic Cancer Cell Line
Full Length RON	20/22 (91%)	7/7 (100%)
RON P5P6	16/22 (73%)	5/7 (71%)

C



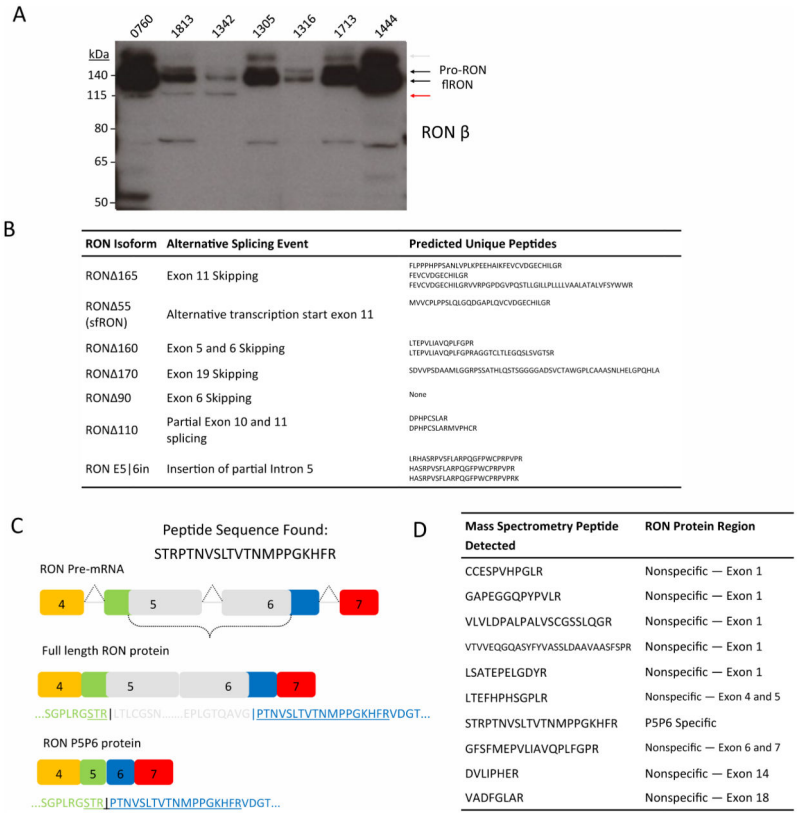
Sample	Mean P5P6 Copies	Percentage of total RON
Nml Panc	0	N/A
0760	211	5.5%
1713	1727	5.3%
1444	3172	2.0%
1316	3389	5.2%
1356	1353	3.8%
1813	1304	7.8%
1305	502	2.6%
1342	12	0.7%
1587	2441	7.2%



Sample	Mean P5P6 Copies	Percentage of total RON
HPDE	727	7.5%
Bx-PC3	1240	3.6%
MiaPaca2	6	3.4%
Panc-1	781	13.2%
AsPC1	2929	4.6%
FG	816	3.8%
79E	879	2.0%
34E	4215	5.7%

Figure 2.

P5P6 is expressed in the majority of pancreatic cancers. A, PCR detects the expression of P5P6 specific transcript in PDX samples. B, The majority of PDX and pancreatic cancer cell lines express the isoform. C, Absolute quantification PCR determined the number of total RON and P5P6 specific transcript copies in each PDX and pancreatic cancer cell line. Both transcripts are overexpressed in all PDX compared to normal pancreas. P5P6 copy number is positively correlated with total RON expression, but with variable overall percentages.



RON Isoform	Alternative Splicing Event	Predicted Unique Peptides
RONΔ165	Exon 11 Skipping	FLPPPHPPSANLVPLKPEEHAIKFEVCVDGECHILGR FEVCVDGECHILGR FEVCVDGECHILGRVVRPGPDGVPQSTLLGILLPLLLVAALATALVFSYWWR
RONΔ55 (sfRON)	Alternative transcription start exon 11	MVVCPLPPSLQLGQDGAPLQVCVDGECHILGR
RONΔ160	Exon 5 and 6 Skipping	LTEPVLIAVQPLFGPR LTEPVLIAVQPLFGPRAGGTCLTLEGQSLSVGTSR
RONΔ170	Exon 19 Skipping	SDVVPSDAAMLGGRPSSATHLQSTSGGGGADSVCTAWGPLCAAASNLHELGPQHLA
RONΔ90	Exon 6 Skipping	None
RONΔ110	Partial Exon 10 and 11 splicing	DPHPCSLAR DPHPCSLARMVPHCR
RON E5|6in	Insertion of partial Intron 5	LRHASRPVSFLARPQGFPWCPRPVPR HASRPVSFLARPQGFPWCPRPVPR HASRPVSFLARPQGFPWCPRPVPRK

Mass Spectrometry Peptide Detected	RON Protein Region
CCESPVHPGLR	Nonspecific — Exon 1
GAPEGGQPYPVLR	Nonspecific — Exon 1
VLVLDPALPALVSCGSSLQGR	Nonspecific — Exon 1
VTVVEQGQASYFYVASSLDAAVAASFSPR	Nonspecific — Exon 1
LSATEPELGDYR	Nonspecific — Exon 1
LTEFHPHSGPLR	Nonspecific — Exon 4 and 5
STRPTNVSLTVTNMPPGKHFR	P5P6 Specific
GFSFMEPVLIAVQPLFGPR	Nonspecific — Exon 6 and 7
DVLIPHER	Nonspecific — Exon 14
VADFGLAR	Nonspecific — Exon 18

Figure 3.

Mass spectrometry confirms the P5P6 protein is expressed in pancreatic cancer**.** A, The β chain of RON is detected by western blot in a representative group of PDX. Pro-RON and full length RON (flRON) bands are prominent (black arrows), however there are different molecular weight bands which may correspond to expressed isoforms (grey and red arrows). B, After trypsin digestion RON isoforms are distinguished by unique peptides using mass spectrometry. C, We discovered a peptide in PDX 1713 which can only be produced by partial exon 5 and partial exon 6 splicing. D, Several other peptides were detected which are specific to RON but not specific to any isoform. This confirms protein expression of P5P6 in pancreatic cancer.

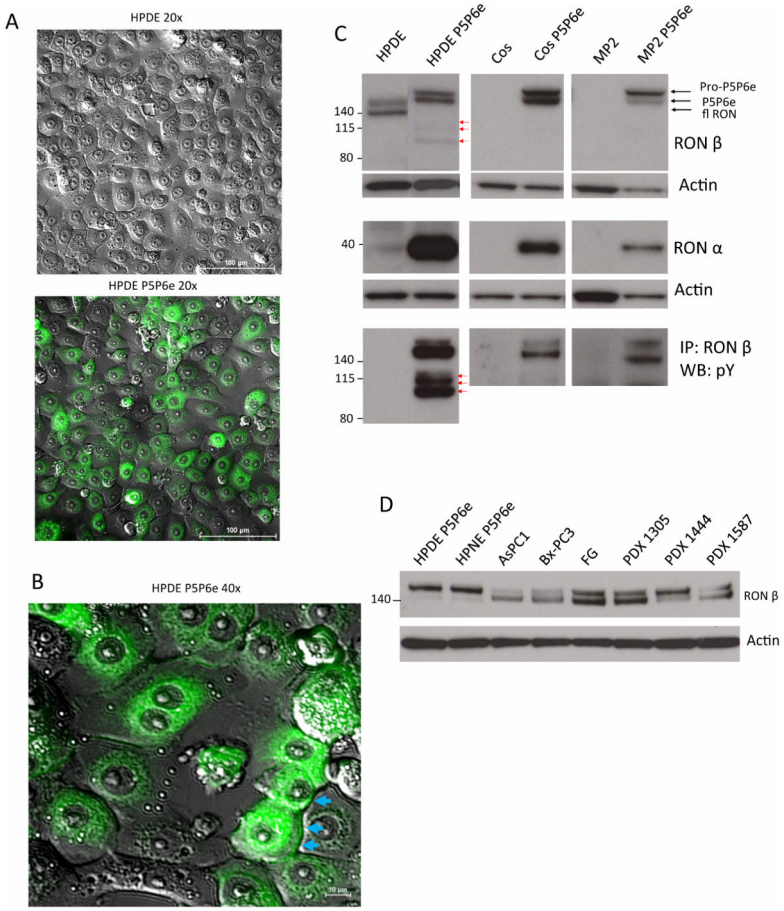


Figure 4.

P5P6 expression in human pancreatic duct epithelial cells. A, The P5P6-eGFP fusion protein is expressed in the HPDE cell line. Live cell imaging shows there is no change in cell morphology but the HPDE P5P6e cell line has a heterogeneous mix of fluorescent cells. B, The P5P6e protein is most abundant in the cytoplasm but some cells display plasma membrane localization (blue arrows). C, P5P6e protein is expressed in all three cell lines and is cleaved from a pro– form into α and β subunits. The isoform is constitutively active in all cell lines, though most heavily phosphorylated in HPDE. In the HPDE cell line, P5P6e is expressed at such a high level that degradation products are detectable and phosphorylated (Red arrows). D, The expression of P5P6e protein in HPDE and HPNE is comparable to total RON expression in pancreatic cancer cell lines (AsPC1, Bx-PC3, FG) and 3 PDX specimens.

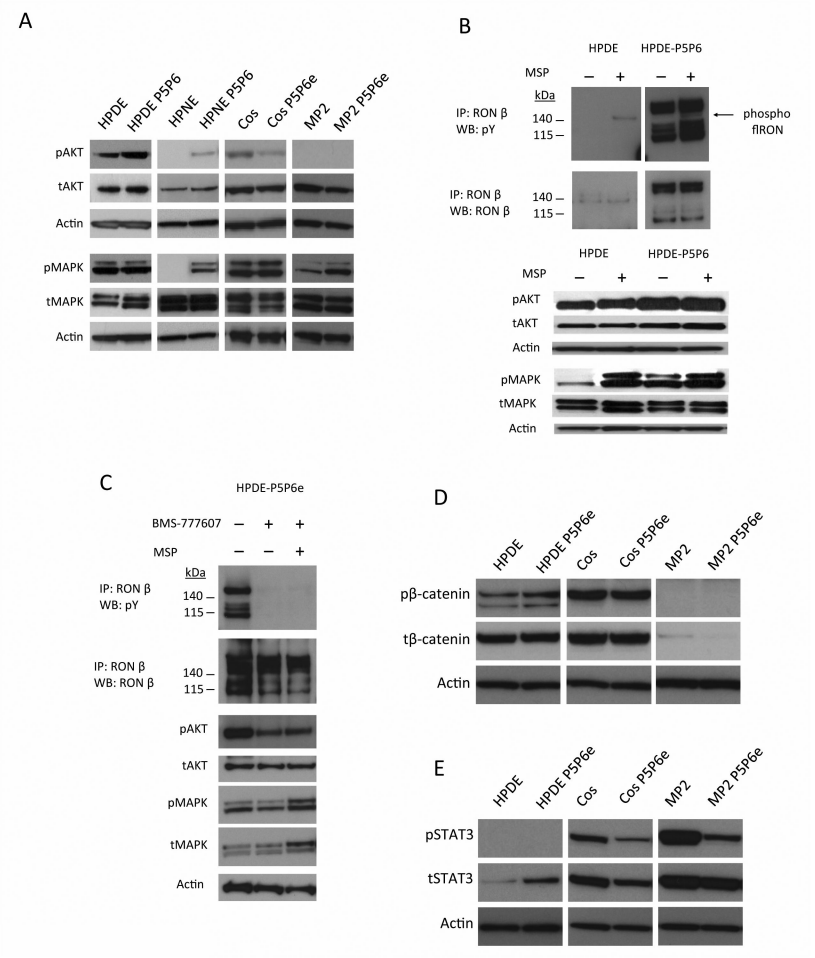


Figure 5.

P5P6 induces oncogenic phenotypes in human pancreatic duct epithelial cells A, P5P6e expression increases AKT phosphorylation in the HPDE cell line and activates both AKT/MAPK in HPNE. B, Stimulation with the RON ligand, MSP (100 ng/mL for 30 minutes) activates full length RON in the HPDE cell line but did not increase P5P6 phosphorylation. MSP stimulation did not change P5P6 downstream signaling. C, Small molecule RON/MET inhibitor BMS-777607 completely inhibited phosphorylation of P5P6 (3 μmol/L for 1 hour). This inhibition could not be overcome with MSP stimulation (100 ng/mL for 30 minutes). There is decreased AKT activation by blocking P5P6 phosphorylation, but there is no effect on the MAPK pathway. D, Mechanism of P5P6e tumorigenesis is distinct from RONΔ160 as there is no accumulation of total β-catenin. E, P5P6 signaling does not activate STAT3 transcription factor pathway in any cell line unlike MSP stimulated full length RON activation.

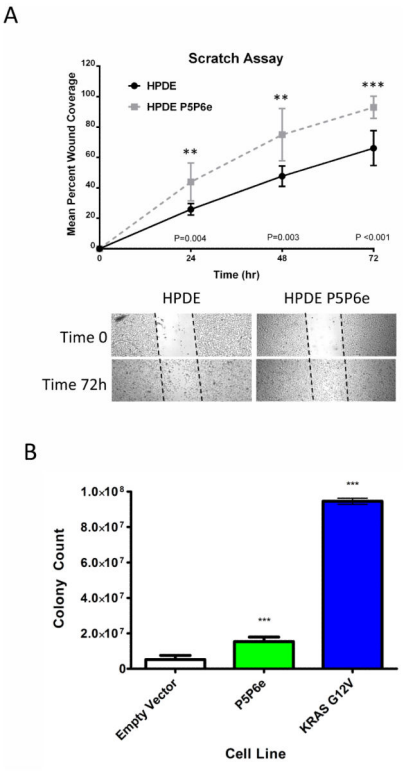


Figure 6.

P5P6 transforms human pancreatic duct epithelial cells. A, Cell motility is significantly increased as a result of P5P6 expression in the HPDE cell lines but not in Cos or MiaPaca2 (Supplemental Figure S2B). B, NIH 3T3 transforming assay showed transformation with expression of P5P6e with significantly more colonies formed than the empty vector. The transforming effect is not as strong as KRAS G12V mutation expression.

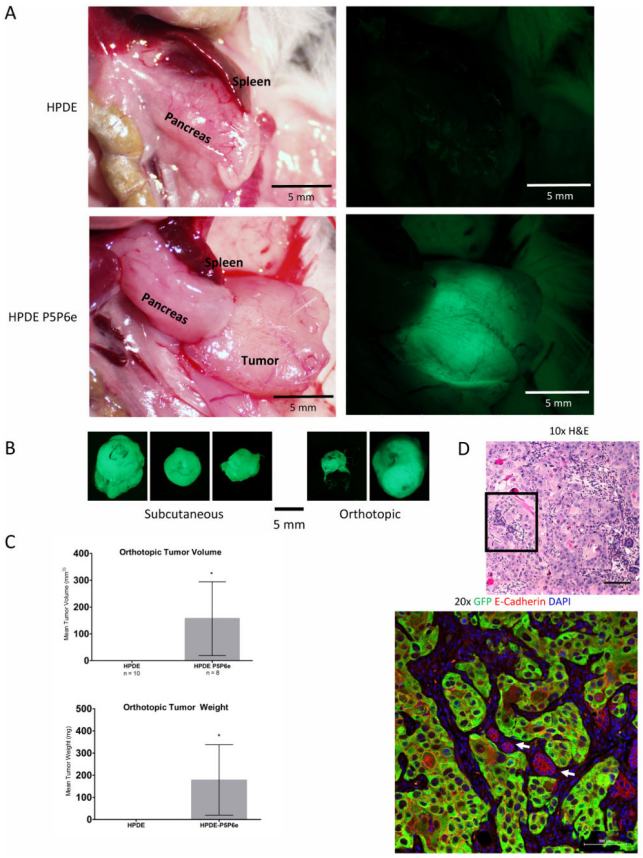


Figure 7.

P5P6 is tumorigenic when expressed *in vivo*. A, HPDE and HPDE P5P6e cells were injected orthotopically and subcutaneously into NSG mice. Only the HPDE P5P6e expressing cell line can form a tumor. B, All tumors expressed the P5P6 eGFP fusion protein and are brightly fluorescent. C, HPDE P5P6e Orthotopic mean tumor volume and weight was statistically significant as 6 of 8 had tumor growth (p-value 0.03). D, H+E and immunohistochemistry shows isoform expressing cells (Green, GFP) are mixed with a large stromal component (Blue, DAPI). HPDE P5P6e cells maintain morphology seen in culture and express E-cadherin (Red). Clusters of residual host pancreas acini are interspersed throughout the tumor (white arrows).